

EFFECTS OF THERAPEUTIC ULTRASOUND ASSOCIATED WITH STATIC STRETCHING ON LONGITUDINAL HISTOMORPHOMETRIC PARAMETERS OF IMMOBILIZED SOLEUS OF RATS



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ABSTRACT

The muscle tissue is endowed with plasticity that adapts to different stimuli. Immobilization causes damage to the musculature including atrophy, loss of muscle strength and extensibility. The stretching and ultrasound treatment modalities are used to speed up muscle repair process as they can increase protein synthesis and improve extensibility. Objective: To compare the use of therapeutic thermal and non thermal ultrasound, associated with stretching, in the remobilization of the soleus muscle of rats subjected to position of muscle shortening on aspects histomorphometric longitudinal muscle. Methods: 28 rats were immobilized for 15 days, later released from the apparatus and divided into four groups: group AG only remobilized by stretching for 10 days and the others were subjected to 10 days of therapeutic intervention 1MHz of ultrasound at 1.0W/cm² (GAUS 1.0), 0.5W/cm² (GAUS 0.5), and 0.2 W/cm² (GAUS 0.2), and further stretching to the soleus. At the end of treatment, the animals were sacrificed and their soleus muscles were removed for later histological analysis of longitudinal parameters (count of sarcomeres). Results: At intragroup analysis on the muscle length, only the group GAUS 0.5 did not present significant difference. The count of sarcomeres in the groups GA and GAUS 0.2 was statistically different. The size of the sarcomeres in both groups had no statistically significant difference. In inter-group analysis both groups had no statistically significant difference for any of the variables. Conclusion: The stretching was insufficient to reverse the effects of immobilization. When associated with therapeutic ultrasound, the dose 0.5 W/cm² recovered muscle length significantly, and the doses 1.0 and 0.5 W/cm² contributed to the significant increase of the number of sarcomeres in immobilized muscles.

Keywords: immobilization, passive stretching, skeletal muscle, sarcomeres.

INTRODUCTION

Although immobilization has been a widely used resource for rehabilitation of injuries, it leads to many deleterious consequences to the musculoskeletal system¹. A short immobilization period (seven days) is already enough for the muscle to suffer morphometric and mechanical adaptations, such as reduction of muscular mass and length, number of sarcomeres, increase of the conjunctive tissue density and reduction of the maximum resistance of muscular rupture²⁻⁴. Increase of conjunctive tissue induces to abnormal crossed ligations of conjunctive tissue fibers, which results in fast muscular stiffness and reduction of range of motion^{1,5}.

Stretching prevents muscular atrophy, proliferation of conjunctive tissue and loss of sarcomeres in series, besides the possibility of activating the protein synthesis and inducing muscular hypertrophy and hyperplasia¹. Some authors state that passive stretching with time of 30 seconds is sufficient to obtain greater mobility, while other authors did not find any effect⁶. Muscular stretching promotes accumulation of slow oxidative RNAm myosin in the fibers termination which help in the synthesis of contractile proteins, in the fast sarcomere union as well as extension of the myofibrils. In special, a large cytoplasmatic space containing polysomes opens between the myofibrils and the sarcolemma of the myotendineous junction of elongated fibers and many myofibrils are found⁷.

The therapeutic ultrasound (TUS) is a resource commonly applied in the disorders of the musculoskeletal system, as well as in the speeding of tissue repair of muscular injuries, increase of cellular proliferation and protein synthesis during healing, besides having effect in the blood circulation. This resource makes use of high-frequency sound waves to deeply penetrate soft tissues⁸⁻¹⁰. The possibility of using different frequencies between 1 and 3MHz is an important measure, since the higher frequencies (3MHz) are more intensely absorbed, becoming more specific for the treatment of superficial tissues, while lower frequencies (1MHz) penetrate more deeply and hence should be used for the deeper tissues¹⁰.

The ultrasound effects are classified in thermal and non-thermal. Ter Haar^{11,12} reports that, among the non-thermal effects of ultrasound, as the mechanical waves of the ultrasound go through the tissues, they cause molecules agitation, which provides greater permeability of the tissue membranes, enabling hence better exchange of its nutrients and catabolites removal. Moreover, the ultrasound non-thermal effects (mechanical) improve the cellular metabolism, besides aiding in the release of adhesions, by the separation of the collagen fibers¹³. Concerning the thermal effects, the tissues are warmed up, which provides countless physiological benefits, especially increase in the blood flow in the area to be treated, increase of the collagen extensibility, decrease of articular stiffness, pain relief and decrease of muscular spasms^{11,12}.

In physiotherapeutic clinical practice, ultrasound and stretching are resources widely used; however, many times we do not know the real effects in cellular level of these modalities, in this case, over the muscular fiber. Considering this fact and the deleterious effects of immobilization on the muscular fibers, it is important to understand their implications in the recovery of the shortened muscle, so that this information can be transferred to the clinical practice.

Thus, the aim of this study was to verify and compare the use of stretching, associated or not to use of therapeutic ultrasound, thermal or non-thermal, in the remobilization of the soleus muscle of rats submitted to muscular shortening position, concerning the longitudinal histomorphometric aspects of these muscles.

MATERIALS AND METHODS

28 albino male Wistar rats with 10 ± 2 weeks of age, obtained in the Central Animal Facility of the State University of Western Paraná (Unioeste) were used. The study was conducted according to the International Guidelines of Ethics in Animal Experimentation¹⁴ and was approved by the Ethics Committee of Animal Studies of the Unioeste. The animals were sorted and kept in polypropylene cages under controlled environmental conditions, with light/dark cycle of 12 hours, with temperature of $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and water and food *ad libitum*.

All animals had the right hinder limb immobilized in maximum plantar flexion, for 15 consecutive days, and were subsequently randomly sorted in four groups with seven animals each for the therapeutic intervention:

GS (Group Stretching): in which, after the immobilization period, the animals were submitted to the static stretching protocol of soleus muscle;

GSUS 1.0 (Group Stretching and TUS of $1.0\text{W}/\text{cm}^2$): group in which the animals were submitted to the treatment protocol with TUS and immediately after the static stretching protocol;

GSUS 0.5 (Group Stretching and TUS of $0.5\text{W}/\text{cm}^2$): similar to the previous group, but with $0.5\text{W}/\text{cm}^2$ dose;

GSUS 0.2 (Group Stretching and TUS of $0.2\text{W}/\text{cm}^2$): similar to the previous groups, but with therapeutic dose of $0.2\text{W}/\text{cm}^2$.

Immobilization protocol

In this study, the immobilization apparatus chosen was the model developed by Coutinho et al.¹⁵, which aims to obtain shortening of the soleus muscle. Therefore, the ankle joint was immobilized at maximal plantar flexion. The shortening position was chosen for causing greater harm to the muscle function¹⁶. The animals were daily observed, during the 15 days of immobilization, in order to identify possible damage to the apparatus. After the immobilization removal, the rats were weighed and submitted to cleaning of the right hinder limb on the region of the triceps surae muscle (soleus).

Ultrasound application protocol

Ultrasound therapy used the machine Sonopulse brandname Ibramed®, with frequency of 1.0MHz and $1.0\text{W}/\text{cm}^2$ dose, $0.5\text{W}/\text{cm}^2$, $0.2\text{W}/\text{cm}^2$, respectively to groups GSUS 1.0; GSUS 0.5 and GSUS 0.2, during three minutes on the soleus region of the right hinder limb for a period of 10 days, with two-day interval at the weekends. A handmade restrainer was used in the immobilization to minimize the stress experienced by the animal.

Stretching protocol

In order to apply stretching to the soleus muscle, the ankle joint was manually kept at maximum dorsal flexion during the entire stretching period, at the limit of tissue tension. The intervention consisted of three sets of 30 seconds, with recovery interval of 30 seconds between sets, during 10 days. Likewise, there was a two-day recovery period at the weekends.

Animals' euthanasia

At the end of the experiment, all animals were weighed and euthanized by guillotine decapitation. Immediately after, the right (treated) and left (control) soleus muscles were isolated for cleaning and weighting on analytical scale (Shimadzu®). Subsequently, the muscles were attached to a styrofoam board in their rest length, for length check using an analog pachymeter (Mitutoyo®) and later preparation of the mountings for histological analysis.

Mounting preparation and histological analysis

After having been attached to the styrofoam boards, the muscles were immersed in formaldehyde (10%) during three hours, for tissue attachment. After this period, they were immersed in nitric acid (30%) for 72 hours, in order to break the conjunctive tissue, and later stored in glycerol solution (50%)¹⁷.

Subsequently, the muscles were placed on a Petri dish and with the aid of an optical lens (Micronal®), nine muscle fibers were isolated with the help of ultrafine tip tweezers. The isolated fibers were then positioned on waxed histological slides. Out of the nine selected fibers, five were used for counting of the number of sarcomeres in series (the ones with the best visual aspect were selected), during $50\mu\text{m}$ in six non-consecutive fields, in a total of $300\mu\text{m}$ of analysis.

An ordinary light microscope (Olympus®), with objective of 40 times, attached to a DCE-s camera was used, with which the images were digitalized. The Image-Pro-Plus 3.0 program was also used to count the sarcomeres in a distance corresponding to $50\mu\text{m}$.

Simple rule of three was used for estimation of the total of sarcomeres in series in the analyzed muscle. The considered variables included soleus muscular length, size and number of sarcomeres in series.

STATISTICAL ANALYSIS

The data obtained were analyzed with the Student's *t* test (for comparison within groups) and ANOVA (for comparison between groups), with Tukey post-test. In all cases, the significance level accepted was of 5%.

RESULTS

Intragroup analysis

Based on the longitudinal histomorphometric parameters of the immobilized soleus muscles of Wistar rats, there was no difference when the left (control) and right (experimental, immobilized and stretched with previous ultrasound $0.5\text{W}/\text{cm}^2$ application) muscles were compared (table 1). Concerning muscular length, in the intragroup analysis, only the GSUS 0.5 group did not present significant difference. Concerning the sarcomeres counting, GS and GSUS 0.2

Table 1. Result of muscular length, sarcomere in series quantity and sarcomere size variables, according to the evaluated group, comparing the right soleus muscle (RSM) and the left soleus muscle (LSM).

	L GS	R GS	p<0,05
Muscle length	2.23 ± 0.14	2.05 ± 0.13*	p = 0.0250
Sarcomeres in series	11359.0 ± 77.640	9988.00 ± 1122.00*	p = 0.0393
Sarcomere size	1.97 ± 0.143	2.06 ± 0.01173	p = 0.2892
	GSUS 1.0 L	GSUS 1.0 R	p<0,05
Muscle length	2.24 ± 0.07	2.07 ± 0.12*	p = 0.0131
Sarcomeres in series	11668.00 ± 975.60	10793.00 ± 745.60	p = 0.1442
Sarcomeres size	1.93 ± 0.13	1.92 ± 0.12	p = 0.9350
	GAUS 0.5 L	GSUS 0.5 R	p<0,05
Muscle length	2.13 ± 0.14	2.03 ± 0.12	p = 0.2016
Sarcomeres in series	11410.00 ± 793.30	10508.00 ± 895.40	p = 0.0728
Sarcomere size	1.86 ± 0.04	1.94 ± 0.12	p = 0.1636
	GSUS 0.2 L	GSUS 0.2 R	p<0,05
Muscle length	2.14 ± 0.09	2.04 ± 0.13*	p = 0.012
Sarcomeres in series	11234.00 ± 500.60	10359.00 ± 782.20*	p = 0.0491
Sarcomere size	1.90 ± 0.07	1.98 ± 0.14	p = 0.3171

* Statistically significant difference.

groups presented significant difference between the right (treated) and left (control) muscles. Finally, concerning the sarcomeres size, none group presented significant difference.

Intergroup analysis

The results shown in the analysis between the different treatment groups (GS; GSUS 0.2; GSUS 0.5 and GSUS 1.0) did not present statistically relevant difference for any of the analyzed variables.

DISCUSSION

The functional length of a muscle is important to influence on its contractile properties¹⁸⁻²⁰ and to determine if the muscle adds or loses sarcomeres^{20,21}.

In the present study, only the immobilized and stretched group with previous therapeutic ultrasound with 0.5W/cm² dose did not present statistically significant difference concerning the quantity of sarcomeres in series and muscular length, when compared with the immobilized and non-immobilized muscles were compared; that is to say, this dose associated with stretching presented positive effects concerning recovery of the deleterious effects caused by immobilization. Costa et al.²² report that the ultrasound produces increase of temperature in the muscular structures, providing muscular relaxation and alteration in viscoelasticity. Such properties may have influenced on the return of the muscular length for this group.

The advantage of the 0.5W/cm² dose may be linked to the presence of non-thermal effects associated with the thermal effects, which would be responsible for the production of an alteration in the permeability of the membrane and stimulation of the transportation of substances, such as the second messengers. These second

messengers stimulate the proliferation of satellite cells, which could make new fibers in case of death of the cell or would help in the repairing of a focal injury⁹. Bertolini et al.²³ analyzed the effects of stretching and ultrasound associated or not, and verified that static passive stretching associated with therapeutic ultrasound of 0.5W/cm² produced alterations only in muscular length at rest, which was increased, corroborating the result of this study.

The application of TUS 1.0W/cm², associated with stretching, was not sufficient to reestablish muscular length after immobilization. According to Garret et al.²⁴, in order to obtain thermal effect with application of ultrasound, a minimum time of application of five minutes is necessary. Thus, the time of ultrasound application in the present study (three minutes) may have not been sufficient to promote the thermal effects expected with the 1.0W/cm² dose.

Rantanen et al.⁸ did not verify regeneration of muscular tissue by application of thermal ultrasound. The same situation was verified in the present study, in which the non-thermal dose of 0.2W/cm² did not recover the muscular length.

The DNA synthesis of the muscle seems to be controlled by its contractile activity^{25,26} and mechanical stimuli such as stretching^{27,28}. Immobilization at shortening position induces to atrophy and tissue protein loss^{29,30}. Moreover, growth promoting factors do not act in the immobilized muscle at shortening position, and hence, loses sarcomeres and suffers atrophy²¹.

The loss of number of sarcomeres in series may be caused by adjustment of the fibers in their extremities, with ideal overlapping of actin and myosin in myofibrils to develop the maximum tension at shortening position^{20,31}. In the present study, it was verified that despite the shortening, there was not reversion of the decrease of quantity of sarcomeres in series derived from the immobilization at shortening position. In previous studies^{32,33}, short-period stretching per se did not normalize the quantity of sarcomeres in series either when compared with the control muscle.

The lack of positive results in the present study concerning stretching may be attributed to the lack of intervals between the interventions and it, considering that other studies^{34,35} suggested that low frequency of stretching would hamper the degeneration and alterations of the muscle fiber. Thus, the time of stretching application (30 seconds), performed here may have not been sufficient. Moreover, the high frequency of application may have not given time for muscular recovery. A suggestion here hence is an analysis with stretching time longer than 30 seconds, as well as days of interval between applications.

In the groups in which stretching associated with TUS 1.0 and 0.5W/cm² was used, significant differences were not observed between right and left limbs concerning the quantity of sarcomeres; that is to say, the effect of reduction of sarcomeres in series consequent of the immobilization was reverted. The possible addition of sarcomeres may have been a result of the proliferation of satellite cells, which agglutinate with the cells of the preexisting muscular fibers⁶.

In the GS and GSUS 0.2W/cm² groups, there was significant difference between the quantity of sarcomeres in series when the right and left sides are compared; that is to say, there was no recovery of the quantity of sarcomeres. Deyne³⁶ analyzed the effect of stretching and contractile stimulation over the

myofibrillogenesis and did not verify stretching as an important factor for the development and maintenance of the muscular sarcomeric structures. When a substance is exposed to a passive force (stretching), it will deform according to the properties of the material, and when a relatively low force is held for a long period of time, the majority of the materials deform in a time-dependent manner. Thus, the duration of the stretching performed in isolation may have been sufficient to promote this deformity⁶.

Increase in length of sarcomere in shortened muscles is due to stretching of the remaining sarcomeres, allowing hence the development of maximal tension of the muscle despite being shortened³¹. The GS, GSUS 0.2, GSUS 0.5 and GSUS 1.0 groups did not present significant difference concerning size of sarcomeres between immobilized and non-immobilized limbs, demonstrating the possible return to pre-immobilization size.

CONCLUSIONS

In the present study, stretching alone was not sufficient to revert the deleterious effects of immobilization. When associated with therapeutic ultrasound, only the 0.5W/cm² dose significantly recovered the muscular length, and the 1.0 and 0.5W/cm² doses contributed to the significant return of quantity of sarcomeres in series to normal values of the muscles submitted to immobilization.

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